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ARTICLE

Carotenoids Modulate Cytokine Production in Peyer's Patch Cells ex Vivo

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This study investigated the effects of carotenoid and capsaicin constituents of *Capsicum* on intestinal immune responses in mice. Peyer's patch (PP) cells were isolated from mice orally administered with capsaicin, or one of three carotenoids (β-carotene, β-cryptoxanthin, or lycopene), at 5 mg/kg/day for 7 consecutive days. Collagenase-separated PP cells were then cultured in the presence or absence of concanavalin A (Con A). PP cells from mice treated with capsaicin, β-carotene, or β-cryptoxanthin all showed significantly enhanced interleukin (IL)-2 and interferon (IFN)-γ production when costimulated with 5 μg/mL Con A, with capsaicin having the greatest effect (approximately two times greater than in normal mice). No increase in the production of IL-2 or IL-4 was observed when PP cells from mice were cultured without Con A. We further tested the combined efficacy of carotenoids and capsaicin on intestinal T-cell cytokine production. Oral administration of capsaicin with β-carotene, both at 5 mg/kg/day for 7 days, increased IFN-γ and IL-2 production in cultured PP cells costimulated with Con A. In contrast, oral administration of β-cryptoxanthin counteracted the stimulatory effect of capsaicin treatment on T-helper cytokine production. Flow cytometric analysis revealed that the population of IFN-γ^+ and IL-4$^+$ cells in PPs from mice administered capsaicin and/or carotenoids did not change, which suggested that the effects of carotenoids and capsaicin on cytokine production were not due to changes in the lymphoid population in PPs. These results indicate that carotenoids and capsaicin, which are common components of foods such as *Capsicum*, mutually modulate T-cell immune responses to exogenous or endogenous inducers such as antigens in PPs, without changing the lymphoid population. Carotenoids modulate the potentiality of cytokine production in T cells or indirectly activate T cells but have no triggering effect such as Con A.

KEYWORDS: Carotenoids; capsaicin; cytokine production; Peyer's patch; *Capsicum*; mice

INTRODUCTION

The gastrointestinal tract arguably provides the most important interface between infants and their new environment. It is the site of greatest exposure to microbial products, potential allergens, and a broad range of dietary nutrients with immunomodulatory properties. These complex enteric exposures play a major role in the maturation of the mucosal immune system and have major implications for the success or failure of subsequent tolerance to foods. There is intense interest in how this oral exposure can be optimized to promote tolerance and prevent allergic disease (*1*). The intestinal immune compartment is tightly regulated, and its antigenic repertoire is independently shaped to accommodate the heavy antigenic load that is characteristic of the gut environment (*2*). Gut-associated lymphoid tissue (GALT) constitutes the largest mass of lymphoid tissues in the human body. Consequently, GALT is also an important element of the total immunological capacity of the host. The regulatory events of the intestinal immune response occur in different physiological compartments; they can be aggregated in follicles and Peyer's patches (PPs) or distributed within mucosa, intestinal epithelium, and secretory sites (*3*). Normally, there are 6−10 visible PPs in the ileum of mice (*4*), and approximately 30 PPs are detectable in human ileum (*5*). The intraepithelial T lymphocytes mainly exhibit a suppressor and cytotoxic [T-helper (Th) 1] phenotype, whereas the lamina propria cells exhibit a helper (β) and inducer phenotype. Antigen presentation in PPs is important in determining systemic immune responses including T- and B-cell-dependent immunity, especially on food allergies or tolerance (*6*, *7*). The immunological hypothesis suggests that reduced exposure to pathogens, foods, or their components early in life results in increased immunoglobulin E (IgE) response to allergens and a subsequent increase in allergic diseases. The mechanism for this increased IgE response has been described as an imbalance of Th1/Th2 cells, reduced activation of Th1 cytokines,

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T-regulatory cells, or failure in appropriate activation of innate effector cells and signaling molecules (*1*). Indeed, we recently reported that oral treatment of cancer and allergic diseases with culture fluid from a medicinal entomogenous fungus, *Paecilomyces tenuipes* (Peck) Samson (or its active glycoprotein component), has a stimulatory effect on T-cell-dependent immune response (*8*, *9*), which enhances interleukin (IL)-2 and interferon (IFN)-γ production in cultured PP cells costimulated with concanavalin A (Con A) (*10*). Furthermore, a traditional Japanese and Chinese herbal remedy, Juzen-taiho-to, which modulates systemic immune reaction, has been shown to enhance production of IL-6 and granulocyte−macrophage colony-stimulating factor in PP cells from C3H/HeJ mice (*11*).

Besides the medical properties, it has been reported that the physiological IFN response is increased by intraduodenal administration of alimentary lectins in rabbits (*12*). Furthermore, splenocytes from mice fed with a mixture of fish oil, specific oligosaccharides, and high proteins/lectins increased levels of IL-2, IL-4, and IFN-γ in response to Con A (*13*). Recently, Requena et al. (*14*) have also reported that oral administration of κ-casein glycopeptide, one of the biologically active components of milk, upregulates the expression of tumor necrosis factor-α mRNA in splenocytes costimulated with Con A. These results indicate that studying the cytokine-modulating activity of medicinal herbs or foods in cultured PP cells is a beneficial approach to elucidate their immunopharmacological functions and mechanisms of oral immune responses to allergies, as well as immunotolerance.

We previously have shown that PP cells prepared from mice treated orally with an ethanol extract of chili pepper (*Capsicum annum* Lin.) or with capsaicin (one of the active constituents of the extract) show enhanced production of Th1 cytokines such as IL-2 and IFN-γ when costimulated with Con A (*15*). It has also been shown that fractions rich in carotenoids isolated from *Capsicum* enhance Th1 cytokine production in PPs (*15*). However, the activity of individual carotenoids on the production of Th cytokines in PPs ex vivo is still unclear.

In this study, we examined the effects of three similar carotenoids derived from *Capsicum* (β-carotene, β-cryptoxanthin, and lycopene) on Con-A-induced Th cytokine production and the stimulatory activity of capsaicin in cultured PP cells ex vivo. PP cells require stimulatory signals to produce Th cytokines in culture; therefore, we used Con A as a mitogen to stimulate T-cell blastogenesis. To the best of our knowledge, this is the first study to evaluate the combined effects of extracts from *Capsicum* on intestinal immune reactions.

MATERIALS AND METHODS

Materials. RPMI-1640 medium, phosphate-buffered saline (PBS), fetal bovine serum (FBS), penicillin, and streptomycin were obtained from Invitrogen (Carlsbad, CA). Con A (type IV), type I collagenase, capsaicin, all-*trans*-β-carotene (C4582, α-carotene-free, ≥95%), all-*trans*-lycopene (L9879, >90%), and all-*trans*-β-cryptoxanthin (C6368, ~98%) (**Figure 1**) were purchased from Sigma-Aldrich (St. Louis, MO). All other reagents were obtained from Wako Pure Chemical Co. (Tokyo, Japan). For analysis of the relative abundance of T cells, B cells, and $CD4^+$ and $CD8^+$ T-cell subsets in PP cells, used were the following monoclonal antibodies (mAbs; Beckman Coulter, Hialeah, FL): anti-CD3−fluorescein isothiocyanate (FITC) (KT3, IgG2a), anti-CD4−FITC (YTS191.1, IgG2b), anti-CD8−phycoerythin (PE) (KT15, IgG2a), and anti-CD19−FITC (6D5, IgG2a). For analysis of the relative abundance of IFN-γ^+ and IL-4^+ Th subsets in PP cells, anti-IL-4−PE (11B11, IgG1) and anti-IFN-γ−FITC (XMG1.2, IgG1) were purchased from BD Biosciences (San Jose, CA). The isotype-matched control Abs used in this experiment were IgG1 conjugated to FITC (BD Biosciences) or PE (BD Biosciences), IgG2a conjugated to PE (Beckman Coulter), IgG2a conjugated to FITC (Beckman Coulter), and IgG2b conjugated to FITC (Beckman Coulter).

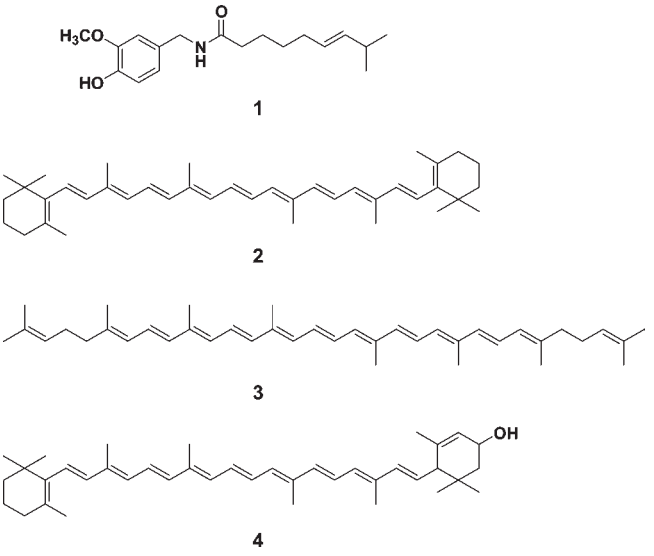

Figure 1. Chemical structures of capsaicin (**1**), β-carotene (**2**), lycopene (**3**), and β-cryptoxanthin (**4**).

Fixation/permeabilization buffer obtained from Beckman Coulter was used to detect Th subsets by the intracellular staining method.

Animals and Test Sample Treatment. Male C57BL/6N mice, 7−10 weeks old, were purchased from Japan SLC (Shizuoka, Japan). Mice were housed in groups of five in plastic cages with a 12 h light/12 h dark cycle and ad libitum access to food and water. Adaptation to these conditions for at least 1 week was allowed before commencing the experiment. The animals were treated according to the "Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences" of The Physiological Society of Japan, and the present experiment was approved by the Institute for Experimental Animals, Kanazawa University Advanced Science Research Centre, Japan (approval number: AP-091373). Capsaicin and each of the three carotenoids were suspended in 5% (v/v) ethanol in 0.1% (w/v) methylcellulose solution, and mice were orally dosed once a day for 7 consecutive days with 50 μL of solution/20 g body weight, which resulted in a dose of 5 mg/kg/day. The final dose of test sample was administered 8 h before PP preparation. The effective dose of 5 mg/kg/day was determined by a previous study (*15*) and a preliminary experiment. Furthermore, we used 5% ethanol in methylcellulose solution as a suitable vehicle for treatment of test samples, because other solvents such as polyoxyethylene sorbitan monolaurate (Tween 20) or DMSO decreased cell viability of PPs after oral administration in a preliminary experiment. Control groups received an equal volume of vehicle instead of test sample.

PP Cell Preparation. Mice were sacrificed with an overdose of ether, and their small intestines were removed and placed into a Petri dish filled with PBS that contained penicillin (100 U/mL) and streptomycin (100 μg/mL), which was kept on ice (*15*, *16*). Visible PPs were carefully dissected out from the wall of the intestine using microscissors under a microscope (7−10 PPs were obtained from each mouse) and were placed in ice-cold complete RPMI-1640 medium that contained 5% FBS, 50 μM 2-mercaptoethanol, 100 U/mL penicillin, and 100 μg/mL streptomycin. To obtain a single-cell suspension, individual PPs were digested with type I collagenase (70 U/mL dissolved in PBS) and incubated for 60 min at 37 °C. After filtration through 200 μm nylon mesh (Becton Dickinson, Oxnard, CA), PP cells were washed three times with complete medium. Cell viability was assessed by trypan blue exclusion. Morphological analysis by characteristic nonspecific esterase and Giemsa staining revealed that >97% of the cells were lymphoid and <2% were monocytes. PP cells (3×10^6 cells/mL) in complete RPMI-1640 medium were seeded in 96-well tissue culture plates (Becton Dickinson) and cultured with or without 5 μg/mL Con A (*15*).

Cytokine Evaluation. To measure cytokine production in cultured PP cells, culture supernatants were collected at specified times (up to 72 h after stimulation with Con A) and stored at −80 °C until use. The levels of IL-2, IL-4, IL-5, and INF-γ in the supernatants were measured by enzyme-linked immunosorbent assay (ELISA) using a commercial kit (eBioscience, San Diego, CA) according to the manufacturer's instructions.

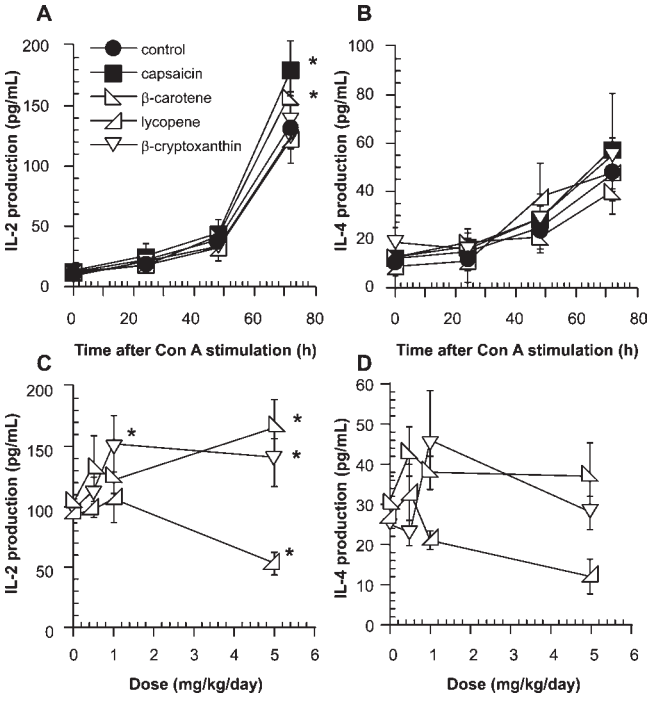

Figure 2. Effects of β-carotene, lycopene, β-cryptoxanthin, and capsaicin on the production of IL-2 and IL-4 in cultured PP cells ex vivo. Mice were treated orally with capsaicin, β-carotene, lycopene, β-cryptoxanthin, or vehicle once daily for 7 consecutive days. PP cells were harvested and cultured with 5 μg/mL Con A for 72 h. Carotenoid doses ranged from 0.5 to 5 mg/kg/day. Culture supernatants were collected at the indicated time, and levels of IL-2 and IL-4 in supernatants were measured using an ELISA kit. (**A** and **B**) Time−course kinetics of IL-2 and IL-4 production in PP cells after Con A stimulation. (**C** and **D**) Dose−response curves of the effects of β-carotene, lycopene, and β-cryptoxanthin on IL-2 and IL-4 production in PP cells 72 h after Con A stimulation. All data are expressed as the means ± SEs of 5−7 mice in each group, $^*p < 0.05$, significantly different from control mice.

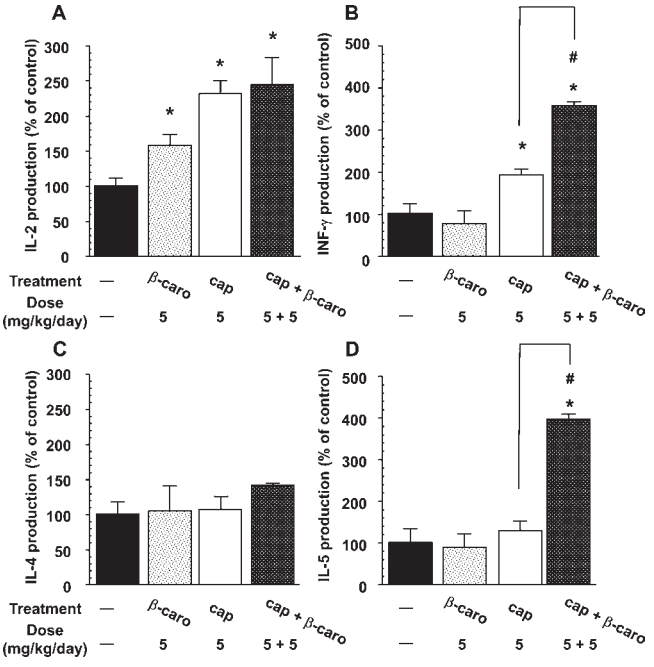

Figure 3. Effects of β-carotene and capsaicin on IL-2, INF-γ, IL-4, and IL-5 production in cultured PP cells ex vivo. PP cells from mice treated orally with β-carotene (β-caro, 5 mg/kg/day), capsaicin (cap, 5 mg/kg/day), β-carotene plus capsaicin (β-caro+cap, 5 + 5 mg/kg/day), or vehicle once daily for 7 consecutive days were collected and cultured in the presence of Con A for 72 h. IL-2 (**A**), INF- γ (**B**), IL-4 (**C**), and IL-5 (**D**) in the culture supernatants were measured using an ELISA kit. All data are expressed as the meand ± SEd of 5−7 mice in each group. $^*p < 0.05$, significantly different from untreated control mice; and $^\#p < 0.05$, significantly different from mice treated with capsaicin alone.

Flow Cytometry. PPs (8/mouse) were individually collected after 7 days of oral administration of the test compounds and dissociated into a single-cell suspension by filtration through a 200 μm nylon mesh. Erythrocytes in PP cell suspensions were removed by lysing in 0.75% (w/v) ammonium chloride buffer (pH 7.6). The resultant PP cells were counted and resuspended in PBS. Cells (1×10^6) were stained with fluorochrome directly conjugated with mAbs (*15*, *17*): anti-CD3−FITC, anti-CD4−FITC, anti-CD8−PE, and anti-CD19−FITC. After 60 min of incubation at 4 °C in the dark, the cells were washed and resuspended in PBS that contained 1 μg/mL propidium iodide (PI). The lymphoid population in the PP cells was analyzed using flow cytometry (FACSCalibur, Becton Dickinson, San Diego, CA) as described below.

For analyzing IL-4$^+$ and IFN-γ^+ T helper subsets, the resultant cells were pipetted directly into a cell-sorting tube containing anti-CD4-PerCP mAb (Becton Dickinson) and incubated at 4 °C in the dark for 30 min. One percent paraformaldehyde (0.5 mL) was added for 8 min to stabilize the mAb−surface antigen complex. Erythrocytes were lysed using 3 mL of 1 × fluorescence-activated cell-sorting lysing solution (Becton Dickinson) for 8 min. After centrifugation for 5 min, the supernatant was aspirated, and 500 μL of 1 × permeabilizing solution (Becton Dickinson) was added to the pellet and incubated for 10 min at room temperature in the dark. After the cells were washed with 3 mL of buffer (1% bovine serum albumin and 0.1% NaN_3 in PBS), anti-IFN-γ−FITC and anti-IL-4−PE were added to the cells, which were incubated for 30 min at 4 °C in the dark. After one final wash, the cells were resuspended in 500 μL of 1% paraformaldehyde and stored at 4 °C until flow cytometric analysis.

Data Analysis for Flow Cytometry. Data were acquired using CellQuest software (Becton Dickinson). Between 10000 and 20000 events were acquired per sample. All data are representative plots derived from analysis of three experimental and three control mice. Mean FITC- and FITC/PE-fluorescence intensities were calculated from fluorescence histograms for the gated population. Gates were set by forward and side scatter to delineate lymphocytes and exclude dead cells; death was confirmed by PI staining. For further analysis of Th cell subsets, anti-CD4-PerCP-positive regions in PI-negative cells were set.

Statistical Analysis. The mean values for cytokine production and ratio of lymphoid or T-cell subpopulations were considered as single data points for analysis of results from at least two independent experiments. Data for cytokine production are expressed as means ± SEs. Statistical significance was determined by Dunnett's multiple test after analysis of variance with comparison to a control group, and the differences were considered significant if $p < 0.05$.

RESULTS

Time−Course Kinetics of Th Cytokine Production in Cultured PP Cells Costimulated with Con A. We examined the time−course kinetics of cytokines IL-2 and IL-4 secreted by PP cells. As indicated in **Figure 2**, production of both cytokines was moderately increased 48 h after Con A (5 μg/mL) challenge, with a further increase after 72 h. No increase in the production of these cytokines was observed when PP cells were cultured without Con A (data not shown). The effects of oral administration of capsaicin or three carotenoids on IL-2 and IL-4 production in cultured PP cells were subsequently examined. PP cells from mice treated orally with 5 mg/kg/day capsaicin showed significantly ($p < 0.05$) increased levels of IL-2 production at 72 h, while capsaicin had no effect on the level of IL-4 production at any time point (**Figure 2A**,**B**). An increase in IL-2 production at 72 h after Con A stimulation was also seen in the case of administration of 5 mg/mg/day β-carotene (**Figure 2A**), although this effect was

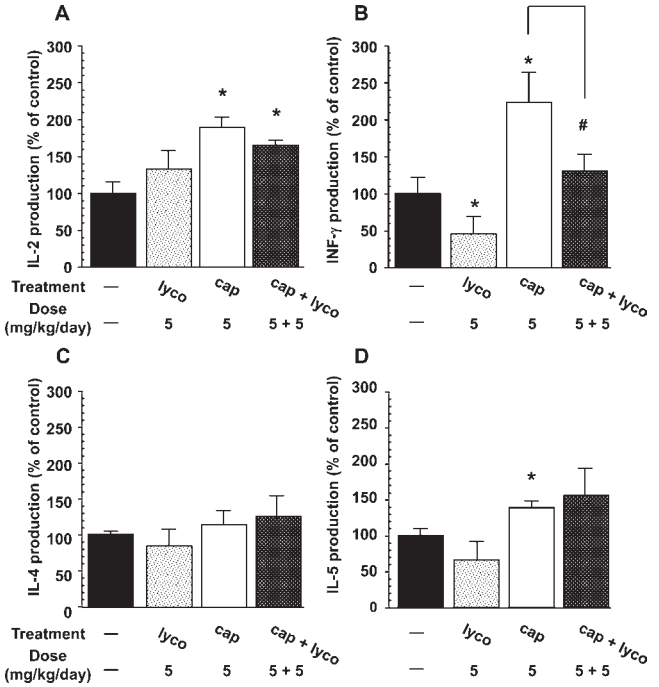

Figure 4. Effects of lycopene and capsaicin on IL-2, INF-γ, IL-4, and IL-5 production in cultured PP cells ex vivo. PP cells from mice treated orally with lycopene (lyco, 5 mg/kg/day), capsaicin (cap, 5 mg/kg/day), lycopene plus capsaicin (lyco+cap, 5 + 5 mg/kg/day), or vehicle once daily for 7 consecutive days were collected and cultured in the presence of Con A for 72 h. IL-2 (**A**), INF-γ (**B**), IL-4 (**C**), and IL-5 (**D**) in the cultured supernatants were measured using an ELISA kit. All data are expressed as the means ± SEs of 5−7 mice in each group. $^{*}p < 0.05$, significantly different from untreated control mice; and $^{\#}p < 0.05$, significantly different from mice treated with capsaicin alone.

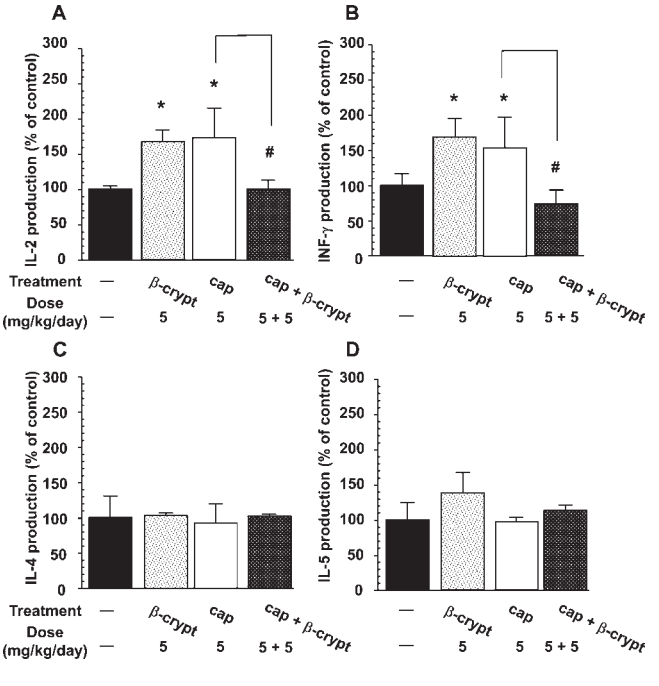

Figure 5. Effects of capsaicin and β-cryptoxanthin on IL-2, INF-γ, IL-4, and IL-5 production in cultured PP cells ex vivo. PP cells from mice treated orally with β-cryptoxanthin (β-crypt, 5 mg/kg/day), capsaicin (cap, 5 mg/kg/day), β-cryptoxanthin plus capsaicin (β-crypt+cap, 5 + 5 mg/kg/day), or vehicle once daily for 7 consecutive days were collected and cultured in the presence of Con A for 72 h. IL-2 (**A**), INF-γ (**B**), IL-4 (**C**), and IL-5 (**D**) in the cultured supernatants were measured using an ELISA kit. All data are expressed as the means ± SEs of 5−7 mice in each group. $^{*}p < 0.05$, significantly different from untreated control mice; and $^{\#}p < 0.05$, significantly different from mice treated with capsaicin alone.

weaker than that of capsaicin (**Figure 2A**). Oral administration of β-cryptoxanthin or lycopene at the same dose (5 mg/kg/day) had no influence on the levels of IL-2 or IL-4 at any time point (**Figure 2A**,**B**). With respect to the dosage level of the carotenoids, oral injection of β-carotene and β-cryptoxanthin at either 1 or 5 mg/kg/day significantly enhanced IL-2 production in cultured PP cells after 72 h, whereas there was no effect on IL-4 (**Figure 2C**,**D**). Conversely, IL-2 production was decreased by administration of lycopene at a dose of 5 mg/kg/day (**Figure 2C**). Higher doses of each of the three carotenoids (10−30 mg/kg) had no effect on the production of Th cytokines in PP cells (data not shown).

Combined Effects of Carotenoids and Capsaicin on Th Cytokine Production by PP Cells ex Vivo. We examined how each of the three carotenoids affected Th1 and Th2 cytokine levels when administered with capsaicin. Oral administration of β-carotene with capsaicin (both at 5 mg/kg) dramatically enhanced the production of IFN-γ and IL-5, with their levels being three times higher than those due to capsaicin alone (**Figure 3B**,**D**). However, there was no difference in levels of IL-2 or IL-4 production in PPs from mice treated with β-carotene and capsaicin as compared with capsaicin alone (**Figure 3A**,**C**). In the case of lycopene treatment, oral administration of this carotenoid alone did not change Th1 and Th2 cytokine levels. PP cells from mice treated with capsaicin and lycopene moderately but significantly increased the production of IL-2 (by 162%), IFN-γ (by 138%), and IL-5 (by 158%); however, the effects of treatment with capsaicin and lycopene together were less than those of capsaicin alone (**Figure 4**). PP cells from mice treated with β-cryptoxanthin alone showed significantly increased IL-2 and IFN-γ [by 163% ($p < 0.05$) and 162% ($p < 0.05$), respectively (**Figure 5A**,**B**)], although oral administration of β-cryptoxanthin had no effect on the production of either IL-4 or IL-5 (**Figure 5C**,**D**). Although capsaicin or β-cryptoxanthin administered individually increased IL-2 and IFN-γ production by a similar level, when mice were administered both simultaneously, there was no effect on either of these cytokines (**Figure 5A**,**B**). There was no change in the production of Th2 cytokines IL-2 and IL-4 when comparing capsaicin-treated and β-cryptoxanthin-treated groups (**Figure 5C**,**D**).

Effects of Carotenoids, Capsaicin, or Carotenoids Plus Capsaicin on Lymphoid Population in Mice. To establish the effects of treatment with capsaicin, carotenoids, or capsaicin + carotenoids on lymphocyte subsets within PPs, PP cells were stained with FITC- or PE-conjugated T, B, and Th subset lineage markers and subjected to flow cytometric analysis. As depicted in **Figure 6**, scatter plot analysis revealed that two typical cell clusters were distributed in PPs (**Figure 6A**), and one major cluster was the PI-negative cell region that identified viable cells (**Figure 6B**). Histogram analysis of a gated PP cell population from a normal mouse showed that the ratios of $CD3^+$ (T), $CD19^+$ (B), $CD4^+$ (Th), and $CD8^+$ (cytotoxic T) cells were 25.8, 66.1, 22.0, and 5.9%, respectively (**Figure 6C**−**E**). Furthermore, IFN-γ^+ (Th1) and IL-4^+ (Th2) subsets of $CD4^+$ Th cells were distributed at 13.9 and 5.5%, respectively (**Figure 6F**).

We performed studies to assess whether carotenoids and/or capsaicin affected relative lymphoid cell population subsets with PPs, in particular the Th subsets. After administration of capsaicin at 5 mg/kg/day, the percentage of $CD3^+$ cells within the total T-cell population decreased by 6%, whereas $CD19^+$ B lymphocytes increased to 72.7% (**Table 1**), a finding that was consistent with our previous study (*15*). In the groups that received carotenoid

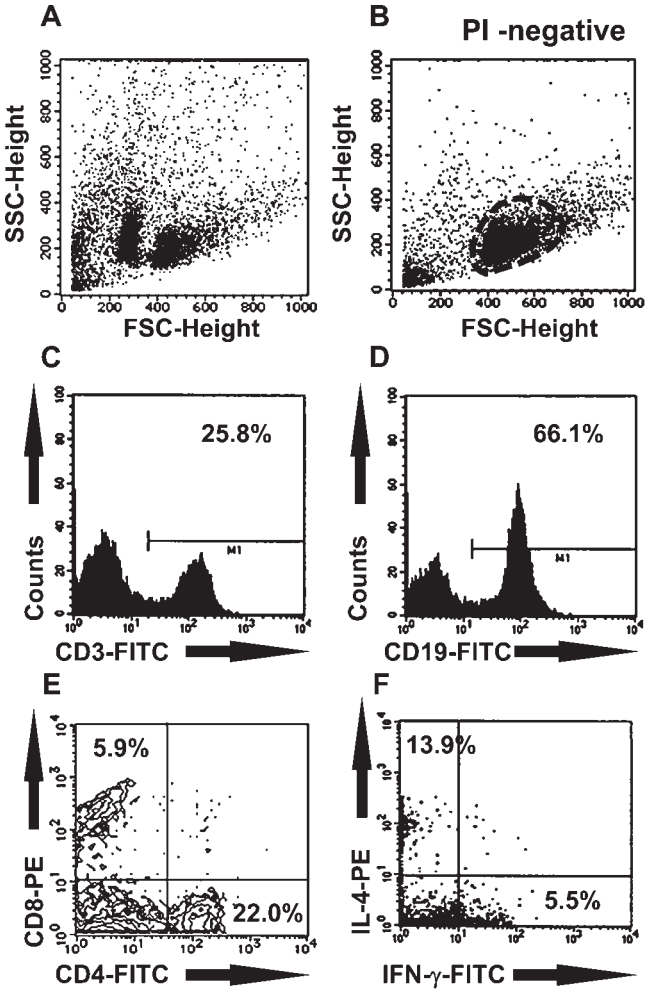

Figure 6. Flow cytometric profile of murine PP cells. PP cells prepared from normal mice were stained with individual lineage-specific FITC- or PE-conjugated markers including anti-CD3, anti-CD19, anti-CD8, anti-CD4, anti-IL-4, and anti-IFN-γ mAbs. To determine cell viability, PP cells were stained with PI. Gates to include viable lymphoid cells for analysis were set by forward and side scatter to delineate lymphocytes and exclude dead cells (**B**); the PI-positive region was used to identify dead cells. (**A**) Dot−plot analysis of unlabeled PP cells; (**B**) dot−plot analysis of PP cells minus excluded dead cell (PI^+ cells); (**C** and **D**) histograms and population of $CD3^+$ and $CD8^+$ cells in PP lymphoid gate; (**E**) quadrant analysis and population of $CD4^+$ and $CD8^+$ cells in PP lymphoid gate; and (**F**) quadrant analysis and population of IL-4^+ and IFN-γ^+ cells on anti-CD4-PerCP mAb-positive cells in PP.

treatment, β-carotene and β-cryptoxanthin weakly decreased the percentage of T cells, and their activity was similar to that of capsaicin. Conversely, oral administration of lycopene at 5 mg/kg/day did not change the percentages of T and B cells, T-cell subset, and Th subset in PP cells (**Table 1**). In addition, the relative population of IFN-γ^+ and IL-4^+ cells that affected Th cytokine production decreased with capsaicin treatment, while the ratio between IFN-γ^+ and IL-4^+ cells was unchanged when compared with control mice (**Table 1**). Individual administration of each of the three carotenoids to the mice treated with capsaicin did not influence the population of T or B cells or the T-cell or Th-cell subsets (**Table 1**).

DISCUSSION

The effects of carotenoids on systemic immune function have been the subject of longstanding discussion. The immunological mechanism of carotenoids is at least partly independent of provitamin A activity because supplementation of a nonpro-vitamin A carotenoid, canthaxanthin, enhances the proliferative activity of T and B lymphocytes in response to Con A and lipopolysaccharide, respectively (*18*). A number of human intervention studies have examined the effect of supplementation with carotenoids on immune function. One such study has described a time-delayed modulation of immune function in healthy men in response to vegetable juice consumption (*19*). In contrast, β-carotene supplementation does not enhance T-cell-mediated immunity in older people (*20*). This finding is consistent with two previously published studies in younger people (*21*) but is contrary to animal and in vitro studies that have suggested a possible beneficial effect of β-carotene supplementation (*22*).

There are some complex mechanisms by which total PP cells can produce Th cytokines in mucosal immune responses. PP mainly contains antigen-specific T and B cells, as well as dendritic cells (DCs). It is hypothesized that immature DCs in PPs constitutively suppress differentiation of Th1 responses to oral antigens via production of immunosuppressive cytokines (IL-10 and transforming growth factor-β), and only following intestinal infection with pathogens do PP DCs trigger Th1 responses by secretion of IL-12 (*23*). Thus, Con A stimulus, a nonspecific T-cell mitogen, is required for detecting Th cytokines by culturing of dissociated PPs. In the present study, oral administration of three carotenoids, β-carotene, β-cryptoxanthin, and lycopene, which are major nutrients in red pepper as well as fruits and vegetables, resulted in modulated Th cytokine production in PPs in response to Con A. The secretion of Th cytokines in PPs from mice treated with individual carotenoids was not affected in the absence of Con A (Supporting Information). These results indicate that carotenoids can modulate the potentiality of cytokine production

Table 1. Effects of Capsaicin, Carotenoids, and Their Combination on the PP Lymphocyte Population in Mice[a]

		%							
treatment	dose (mg/kg/day)	$CD3^+$	$CD19^+$	$CD4^+$	$CD8^+$	IL-4^+	INF-γ^+	INF-γ/IL-4 ratio	*n*
control		32.5	64.6	20.4	5.8	12.9	6.1	0.47	3
capsaicin	5	26.3	72.7	17.8	6.1	9.1	4.2	0.46	3
β-carotene	5	28.5	72.2	18.0	6.3	10.2	5.2	0.51	3
lycopene	5	31.0	65.9	22.1	7.1	14.2	6.9	0.48	3
β-cryptoxanthin	5	23.1	74.3	19.3	6.7	13.0	5.9	0.45	3
capsaicin + β-carotene	5 + 5	21.9	74.7	15.1	9.2	11.0	5.3	0.48	3
capsaicin + lycopene	5 + 5	29.7	66.8	20.5	7.6	12.7	5.8	0.45	3
capsaicin + β-cryptoxanthin	5 + 5	23.7	71.3	16.5	5.8	11.8	5.0	0.42	3

[a] Capsaicin, one of three carotenoids, or capsaicin + each carotenoid were orally administered to mice once a day for 7 consecutive days. PP cells (2×10^6 cells) were collected 8 h after the final administration and stained with lineage-specific FITC- or PE-conjugated mAbs. Data are expressed as the means of the individual lymphoid lineage of three mice. This experiment was repeated twice and showed similar results.

in T cells, or indirectly activate T cells, but have no triggering effect such as that of Con A.

Furthermore, the three carotenoids investigated here showed different effects on production of Th cytokines in PP cells. Oral administration of lycopene at 5 mg/kg/day significantly suppressed IFN-γ production and also exhibited a tendency to suppress IL-4 production in cultured PP cells. In vitro administration of lycopene has been reported to inhibit IL-2 and IL-10 in cultured human peripheral blood mononuclear cells from healthy human subjects (*24*). In contrast, lycopene-treated DCs are poor stimulators of naïve allogeneic T-cell proliferation and induce lower levels of IL-2 in responding T cells (*25*). Our data support these reports and suggest that the in vivo suppression of the immune response by lycopene is in part due to the inhibition of cytokine production by PPs.

In contrast to lycopene, β-cryptoxanthin and β-carotene, which are metabolites of lycopene in carotenoid biosynthesis (*26*), accelerate Th1 cytokine production in cultured PP cells. However, oral administration of β-cryptoxanthin resulted in increased INF-γ production, while β-carotene did not.

In vitro and in vivo experimental studies of the effects of β-carotene on immune responses, including T-cell function, have been reported (*27*). However, the immunopharmacological activity of β-cryptoxanthin, which belongs to the xanthophyll family of oxygen-containing carotenoids, remains to be clarified. In the case of β-carotene, Seifter et al. (*28*) have reported a marked stimulatory action on the growth of the thymus gland and a large increase in the number of thymic small lymphocytes. Increased numbers of Th and T-inducer lymphocytes have been reported in adult humans given oral β-carotene supplementation (*29*, *30*). The number of lymphoid cells with surface markers for natural killer cells and IL-2 and transferrin receptors is increased substantially in peripheral blood mononuclear cells from individuals supplemented with β-carotene (*30*, *31*). Recently, it has been shown that splenocytes from mice fed with β-carotene produce more IL-2 and IFN-γ than those from control mice (*22*). These reports are consistent with our data on the induction of Th1 cytokine production in PP cells from mice-treated with β-carotene. A novel finding is that IL-2 production in PP cells costimulated with Con A was selectively enhanced by administration of β-carotene. It is still unclear why oral administration of β-cryptoxanthin potentiates production of IL-2, but not IFN-γ, in cultured PP cells. Indeed, although the cell lineage is different, β-cryptoxanthin has been reported to have different activity on the uptake and secretion of carotenoids on Caco-2 intestinal cells as compared with β-carotene in an in vitro study (*32*).

We previously have demonstrated that oral administration of capsaicin modulates Th cytokine production in cultured PP cells without changing the lymphoid population (*15*). Hence, we measured the effect of oral administration of three carotenoids on populations of lymphoid and T-helper subsets in PPs using flow cytometric analysis. None of the three carotenoids that we tested, β-carotene, lycopene, and β-cryptoxanthin, at a dose of 5 mg/kg/day (sufficient to elicit Th cytokine production) altered lymphoid population or Th subsets. These results clearly indicate that β-carotene, β-cryptoxanthin, and lycopene accelerate and/or suppress Th cytokines in PPs, without any alteration of relative T lymphoid numbers.

We have previously shown that capsaicin and a carotenoid-rich fraction, both derived from *Capsicum*, enhance Th1 cytokine production in PP cells (*15*). In the current study, having established the individual effects of β-carotene, β-cryptoxanthin, and lycopene in a model system, we investigated the interaction of these three carotenoids with capsaicin by administering them to capsaicin-treated mice. Orally administered β-carotene dramatically potentiated capsaicin-induced IL-2, IFN-γ, and IL-4 production without changing the population of Th1/Th2-cell subsets, while β-cryptoxanthin and lycopene decreased these effects of capsaicin. Thus, carotenoids and capsaicin derived from *Capsicum* can exhibit synergistic, additive, or counteracting effects on intestinal immune responses. To elucidate the potential effects of these carotenoids on intestinal immune response, including food allergies or tolerance, we are currently investigating the activity of carotenoid analogues (e.g., capsanthin, lutein, or astaxanthin) and all-*trans*-retinoic acid (vitamin A) on Th cytokine production in PPs ex vivo.

In the current study, we showed that carotenoids have the potential to modulate Th cytokine production, without quantitatively changing the Th subset in PP cells ex vivo, thereby promoting an intestinal or systemic T-cell immune response. Furthermore, three carotenoids (β-carotene, β-cryptoxanthin, and lycopene, all derived from *Capsicum*) affect capsaicin-induced cytokine production when administered with capsaicin. Carotenoids modulate the potentiality of cytokine production in T cells, or indirectly activate T cells, but have no triggering effect such as Con A does.

Supporting Information Available: Effects of β-carotene, lycopene, β-cryptoxanthin, and capsaicin on production of IL-2 and IL-4 in cultured PP cells without Con A-stimulation. This material is available free of charge via the Internet at http://pubs.acs.org.

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